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(54) Title: METHOD FOR DETECTING PRE-CANCEROUS OR CANCEROUS CELLS USING P90 ANTIBODIES OR PROBES

(57) Abstract

The invention provides a method of diagnosing cancer by determining the expression level or gene amplification of p53 and dm2, whereby an elevated level of either p53 or dm2 or both p53 and dm2 indicates a cancer diagnosis. Furthermore, the invention provides a method of predicting the progress of cancer by determining the expression level or gene amplification of p53 and dm2, whereby an elevated level of either p53 or dm2 or both p53 and dm2 indicated a poor prognosis.

METHOD FOR DETECTING PRE-CANCEROUS OR CANCEROUS CELLS USING P90 ANTIBODIES OR PROBES

This application is a continuation-in-part of PCT Application No.   , filed June 25, 1993, which is a continuation-in-part of U.S. Serial No. 08/018,649,

5   filed February 17, 1993, which is a continuation-in-part of U.S. Serial No. 07/904,766, filed June 26, 1992, which in turn is a continuation-in-part of U.S. Serial No. 07/730,185, filed July 12, 1991, which in turn is a continuation-in-part of U.S. Serial No. 07/543,963 filed

10 June 27, 1990, all of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

15   Mutations of proto-oncogenes in somatic cells are increasingly being recognized as significant in the induction of human cancers. Some examples of oncogenes formed by such mutations include: neu, fes, fos, myc, myb, fms, Ha-ras, and Ki-ras. The mutations that convert proto-

20 oncogenes to oncogenes are often point mutations. Much needs to be learned in order to understand how oncogenes and their expression products function to transform normal cells to cancer cells.

25   Oncogenes are generally believed to act in a dominant fashion. This is generally considered to mean that the conversion of a proto-oncogene to an oncogene results in the acquisition of a new function, i.e., enhancing transformation.

30

A different type of mutation associated with cancer occurs when a tumor suppressor gene is altered in a way that causes the product of the gene to lose its tumor suppressor function. An example of such a tumor suppressor gene is the

retinoblastoma susceptibility gene, Rb.   Tumor suppressor
genes are sometimes called recessive oncogenes, although,
strictly speaking, the products of tumor suppressor genes do
not contribute to tumor formation.   The phenotype is
5   recessive since, when both alleles are mutated, the absence
of a tumor suppressor gene results in an enhancement of
tumorigenesis.

A gene product that exhibits some properties of both a
10   dominant and a recessive oncogene is the 53kd
phosphoprotein, p53. Evidence is growing that mutations in
the p53 gene is associated with a large number of many types
of cancers.   For example, Iggo et al., Lancet 335, 675-679
(1990) has expressed the opinion that p53 is the most common
15   proto-oncogene to undergo mutation in lung cancers.

Much of what is known about p53 has been derived from
studying the effect of transfecting wild-type and mutant
murine p53 in rat embryo fibroblast cells.   This work has
20   been reviewed by Levine et al., "The P53 Proto-Oncogene And
Its Product," in Common Mechanisms Of Transformation By
Small DNA Tumor Viruses, L. Villarreal, ed., American
Society for Microbiology, Chapter 2 (1989); Hinds et al.,
ibid, Chapter 7; and Levine, BioEssays 12, 60-66 (1990).
25

The p53 gene appears to be involved in transcriptional
control (Fields, S. & Jang, S. K.   (1990) Science 249,
1046-1049; Raycroft, L., Wu, H. & Lozano, G. (1990) Science
249, 1049-1051; and Levine, A. J., Momand, J. & Finlay, C.
30   A. (1991) Nature 351, 453-456) and may act as a regulatory
check point in the cell cycle, arresting cells in the G-1
phase (Martinez, J., Georgoff, I. & Levine, A. J.   (1991)
Genes Dev. 5, 151-159; Hupp, T. R., Meek, D. W., Midgley, C.
A. & Lane, D. P.   (1992) Cell 71, 875-886; and Yin, Y.,
35   Tainsky, M. A., Bischoff, F. Z., Strong, C. C. & Wahl, E. M.

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(1992) *Cell* **70**, 937-948). Genetic alterations of the p53
gene, such as intragenic mutations, homozygous deletions,
and structural rearrangements, are frequent events in human
cancer (Vogelstein, B. & Kinzler, K.   (1992) *Cell* **70**,
5   523-526; Baker, S. J. et al. (1990) *Cancer Res* **50**,
7717-7722; Mori, N. et al. (1989) *Cancer Res* **49**, 5130-5135;
Lee, J. H. et al. (1990) *Cancer Res* **50**, 2724-2728; Varley,
J. M. et al. (1991) *Oncogene* **6**, 413-421; Presti, J. C. et
al. (1991) *Cancer Res* **51**, 5405; Dalbagni, G., et al. (1993)
10   *Diagnostic Molecular Pathology* **2**, 4-13).   These altered
patterns of p53 either reduce or inhibit the activity of
functional homotetramer units (Stenger, J. E., et al. (1992)
Mol Carcinog **5**, 102-106; Sturzbecher, H. W., et al. (1992)
Oncogene **7**, 1513-1523).   Mutant p53 proteins have a
15   prolonged half-life and retarded degradation, yielding
accumulation of inactive complexes and self-aggregatory
molecules in the nuclei of tumor cells (Sturzbecher, H. W.
et al. (1987) *Oncogene* **1**, 201-211; Halevy, O. et al. (1989)
Mol Cell Biol **9**, 3385-3392),
20

In humans, germ-line mutations of the p53 gene have
been characterized in members of families affected with the
Li-Fraumeni syndrome, a rare autosomal dominant trait that
predisposes these individuals to develop a variety of
25   tumors, including soft tissue sarcomas (Li, F. P. &
Fraumeni, J. F. (1969) *Ann Intern Med* **71**, 747-752; Malkin,
D. et al. (1990) *N. Engl. J. Med.* **250**, 1233-1238).   More
recently, p53 germ-line mutations were also detected in
cancer patients with no apparent family history of cancer
30   (Toguchida, J. et al. (1992) *N. Engl. J. Med.* **326**,
1301-1308), as well as a subset of patients presenting with
a second primary neoplasm (Malkin D., et al. (1992) *New Eng
J Med* **326**, 1309-1315).   In addition, somatic mutations of
the p53 gene have been reported to occur in soft tissue
35   sarcomas (Stratton, M. R., et al. (1990) *Oncogene* **5**,

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1297-1301; Mulligan, L. M., et al. (1990) *Proc Natl Acad Sci USA* **87**, 5863-5867; Toguchida, J. et al. (1992) *Cancer Res* **52**, 6194-6199; Drobnjak, M. et al. (1993) Submitted; Latres, E., et al. (1993) Submitted).

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Another gene that has been identified as having oncogenic potential is the murine double minute-2 (mdm2) gene (Fakharzadeh et al., (1991) The EMBO Journal **10**(6):1565-1569). The sequences of the murine and human

10 mdm2 genes and proteins are known (Fakharzadeh et al., The EMBO Journal **10**(6):1565-1569 (1991); Oliner, J.D. et al. (1992) *Nature* **358**:80-83; and Cahilly-Snyder et al., Somatic Cell and Molecular Genetics, **13**(3):235-244 (1987)). The sequence is evolutionarily conserved among species including

15 mouse, rat, hamster and human genomes (Cahilly-Snyder et al., Somatic Cell and Molecular Genetics, **13**(3):235-244 (1987)).

The mdm2 gene is also referred to in the literature as

20 MDM2, *MDM2* and hdm2 (the human homologs) and *mdm2* (murine). The mdm2 gene product, which is a 90 kD phosphoprotein, is referred to in the literature as p90, which refers to both murine and human homologs, and MDM2, which is the human homolog. The p90 protein is described in applicants'

25 related publication, Levine et al., International Application No. PCT/US91/04608, filed June 27, 1991. Where dm2 is used throughout this application, it is meant to encompasses the various terms in the literature for the mdm2 gene and protein, including homologs among all species.

30

There is evidence for *MDM2* amplification and MDM2 (gene product) overexpression in both osteo- and soft tissue sarcomas (Oliner, J. D. et al. (1992) *Nature* **358**, 80-83; Ladanyi, M. et al. (1993) *Cancer Res* **53**, 16-18; Leach, F. S.

35 et al. (1993) *Cancer Res* **53**, 2231-2234).

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The human homolog of the mdm2 gene, called the hdm2 gene or *MDM2* or MDM2, has been cloned and mapped to the long arm of chromosome 12 (12q13-14) (Oliner et al. 1992.

5 Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature <u>358</u>:80-83). This region contains two genes, SAS and GLI, previously found to be amplified in osteo- and soft tissue sarcomas. The SAS gene codes for a protein of unidentified function. It was isolated from a

10 malignant fibrous histiocytoma (MFH), and was shown to be amplified in MFH and liposarcomas (Turc-Carel, C. et al. (1986) *Cancer Genet Cytogenet* **23**, 291-299; Meltzer, P. S. et al. (1991) *Cell Growth Diff* **2**, 495-501). The GLI gene codes for a DNA-binding zinc finger protein. Even though it was

15 originally isolated from a glioblastoma, it has also been reported to be amplified in a rhabdomyosarcoma and an osteosarcoma (Kinzler, K. et al.(1984) *Science* **236**, 70-73).

Prior to the present invention, it was known that

20 mutated p53 is associated with cancer. Futhermore, it was known prior to the present invention that overexpression of mdm2 is associated with tumors. However, there has been no disclosure prior to the present invention of the relationship between altered p53 and dm2 genes and their

25 altered patterns of expression in cells and how to utilize this relationship to diagnose as well as to determine the clinical relevance or prognoses of cancer patients. An objective of the subject invention is to utilize the relationship between p53 and dm2 overexpressed or amplified

30 genes to diagnose cancer as well as to determine the prognoses of cancer patients.

SUMMARY OF THE INVENTION

35   The subject invention provides a method of diagnosing

cancer by determining the level of p53 and dm2 in a
biological sample, whereby an elevated level of either p53
or dm2 or both p53 and dm2 indicates a cancer diagnosis.

5   A further objective of the invention has been met by
providing a method of predicting the progress of cancer by
determining the level of p53 and dm2 in a biological sample,
whereby an elevated level of either p53 or dm2 or both 53
and dm2 indicates a poor prognosis.

10

The invention further provides a method of classifying
a biological sample into one of three groups, the method
comprising determining whether the level of either p53 or
dm2 in the sample is abnormally elevated, whereby the first

15   group comprises no abnormal elevation of either the level of
p53 or dm2, the second group comprises abnormal elevation of
the level of p53 and no abnormal elevation of the level of
dm2 or abnormal elevation of the level of dm2 and no
abnormal elevation of the level of p53, and the third group

20   comprises abnormal elevation of the level of both p53 and
dm2.

The invention further provides a method of detecting in
a biological sample cancer cells or cells at risk of

25   becoming cancerous or pre-cancerous, wherein the cells
contain at least one normal p53 allele.   The method
comprises determining whether the level of dm2 in the
biological sample is abnormally elevated, whereby an
elevated level of dm2 in the biological sample in comparison

30   to normal biological samples indicates cancer cells or cells
at risk of becoming cancerous or pre-cancerous.

The invention also provides an isolated p53/dm2 protein
complex and antibodies to the dm2 protein.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1:   This figure indicates epitopes of the p90 protein that react with the anti-p90 monoclonal antibodies
5 of the invention.   The anti-p90 monoclonal antibodies produced by hybridomas 1F5, 6C10, 1D6, 4B2, 2E12, 3F3, 3G5, 3F8, 6H7, 2A9, 3G9, 1D11, 2A10, 1G2, 4B11 and 5B10 are indicated beneath the p90 amino acid epitope map at locations of the map indicating the epitopes with which such
10 antibodies react.

Figure 2: This graph shows the overall survival of 211 patients with soft tissue sarcomas over a period of 90 months.
15

Figure 3: This graph shows p53/mdm2 elevated levels and survival in the group of 211 patients (see Figure 2) with soft tissue sarcomas. The phenotypic categories shown in the graph are as follows: Group A: dm2-/p53- (neither dm2
20 nor p53 had elevated levels); Group B: dm2+/p53- and dm2-/p53+; and Group C: dm2+/p53+ (elevated levels of both dm2 and p53).

Figure 4: This photograph   illustrates   immunostaining
25 patterns using anti-dm2 and anti-p53 antibodies. The control group refers to the top two slides. The top left slide shows a staining pattern of a 3T3-Balb/c cell line, which is dm2-. The top right slide shows a staining pattern of 3T3-DM cell line, which is dm2+. The middle two slides
30 show a staining pattern of human tumor tissue sections taken from the same patient. The middle left slide shows a p53- staining pattern, and the middle right slide shows a dm2+ staining pattern (corresponding to the Group B subset p53-/dm2+ subset described below). The bottom two slides show
35 a staining pattern of human tumor tissue sections taken from

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a different patient.   The bottom left slide shows a p53+
staining pattern, and the bottom right slide shows a dm2+
staining pattern (corresponding to Group C described below).

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DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS:

10 **p53**:

For the purposes of the present specification, the term
"wild-type" p53 means the nucleotide or amino acid sequence
reported by Matlashewski et al., EMBO J. <u>13</u>, 3257-3262
(1984); Zakut-Houri et al., EMBO J. <u>4</u>, 1251-1255 (1985); and
15 Lamb and Crawford, Mol. Cell. Biol. <u>5</u>, 1379-1385 (1986).
The sequences are available from GenBank.   Wild-type p53
includes a proline/arginine polymorphism at amino acid 72
and the corresponding nucleotide polymorphism.

20   Mutations of wild-type p53 genes and proteins indicate
pre-cancer and cancer states.   A pre-cancer cell is a cell
that typically has one normal p53 allele and one mutated p53
allele.   For example, the mutation may be a point mutation.
In a cancer cell, both p53 alleles are usually mutated.   For
25 example, one mutation may be a point mutation, and the other
mutation may be a deletion of all or a significant part of
the p53 gene.

dm2:

30   The dm2 of this invention refers to a family of
proteins that includes a phosphoprotein of 90 kD (p90), its
fragments or products, including proteins p85 (85 kD), p76
(76 kD), p74 (74 kD) and p58-57 (58 kD and 57 kD) (p57 is
the murine equivalent to the rat p58), and homologs or
35 analogs thereof.   The p53 protein co-immunoprecipitates with

the dm2 protein.   The dm2 protein includes the known,
sequenced   murine   double   minute   2   (mdm2)   90   kD
phosphoprotein. Furthermore, the term "dm2" refers to genes
encoding for the family of dm2 proteins described above.
5   The sequence of the mdm2 gene and protein is disclosed by
Fakharzadeh et al., The EMBO Journal   10(6):1565-1569
(1991); and Cahilly-Snyder et al., Somatic Cell and
Molecular Genetics, **13**(3):235-244 (1987)).   Sequences
homologous to the p90 dm2 are present in the genomes of
10   several species including human ("hdm2").   The human gene
has   been   sequenced   and   has   a   molecular   weight   of
approximately 90 kD (Oliner, J.D. et al. (1992) *Nature*
358:80-83), as well as rat, mouse and hamster.   In a
preferred embodiment, the dm2 gene and protein are human.
15

        dm2 proteins of approximately 90 kD clearly, and p58
most likely, binds to p53. 3T3DM cells derived from Balb/c
3T3 overproduce the five mdm2 protein species in response to
the amplified mdm2 gene copy.
20

Elevated Levels of dm2 and/or p53 (dm2+ and/or p53+):

        For the purposes of this specification, elevated levels
of dm2 and/or p53 in a cell may indicate dm2 or p53 gene
25   amplification,   or   of   dm2   or   p53   protein   product
overexpression or accumulation in a biological sample, such
as a cell.   Elevated levels of dm2 or p53 protein are a
measure of total dm2 or p53 protein in a biological sample,
preferably a cell, whether free protein or in a complex. In
30   some cases, the dm2 protein elevated levels in the absence
of   dm2   amplification   indicates   the   formation   of
heterodimers/heterotetramers between dm2 and mutated p53
products.   Mutant p53 proteins have a prolonged half-life
and retarded degradation, and therefore accumulate in the
35   cell nuclei. p53 missense mutations represent the majority,

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at least about 85%, of the p53 mutations detectable by immunochemistry. However, in some cases, wild-type p53 genes and proteins are detected at elevated levels in tumor tissue.

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An elevated level of dm2 and/or p53 in a biological sample in comparison to normal biological samples indicates a cancer cell or cell at risk of becoming cancerous or pre-cancerous. A biological sample may include but is not 10 limited to, tissue extracts, cell samples or biological fluids such as lymph, blood or urine.

The subject invention provides a method of classifying a biological sample into one of three groups, the method 15 comprising determining whether the level of either p53 or dm2, or both p53 and dm2, in the sample is abnormally elevated. The first group, Group A, comprises no abnormal elevation of either the level of p53 or dm2 (p53-/dm2-); the second group, Group B, comprises abnormal elevation of the 20 level of dm2 and no abnormal elevation of the level of p53 (p53-/dm2+), or abnormal elevation of the level of p53 and no abnormal elevation of the level of dm2 (p53+/dm2-); and the third group, Group C, comprises abnormal elevation of the level of both p53 and dm2 (p53+/dm2+).

25

The invention demonstrates that classifying altered patterns of dm2 and p53 expression is clinically significant for the diagnosis and prediction of the clinical outcome of patients with various types of cancer. Such cancers include 30 sarcomas, carcinomas and leukemias or lymphomas. Particularly, such cancers include sarcomas, such as soft tissue sarcomas and osteogenic sarcomas. In another embodiment, the cancers include bladder cancers. Other embodiments include, but are not limited to, colorectal, 35 lung, ovarian, cervical, adrenal cortex, bone, breast,

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brain, chronic myelocytic leukemia, and chronic myelogenous leukemia. Therefore, the invention provides a method of assessing a subject's prognosis by obtaining a biological sample from pre-cancer tissue or a tumor of the subject, and

5 determining to which of the three groups, A, B, or C, the biological sample belongs. Of the three categories, the third group indicates the worst prognosis and the first group indicates the best prognosis.

10

Group C (p53+/dm2+):

The invention provides a method for detecting a cancer cell or cell at risk of becoming cancerous or pre-cancerous,

15 wherein the cell contains at least one mutant p53 allele, by determining whether elevated levels of dm2 are present.

The invention unexpectedly demonstrates that elevated levels of dm2 protein act synergistically with elevated

20 levels of p53 protein, including mutant as well as overexpressed wild-type forms of p53 protein, which may be detected as elevated levels of p53, i.e., the p53+/dm2+ group (see Figure 4), resulting in clinicopathological variables of poor prognosis, including survival and tumor

25 progression. Of the three groups, Group C indicates the worst prognosis. Example 2 and Figure 3 show that the immunological detection of abnormally elevated levels of dm2 and p53 in the same tumor sections occurred in a group with very poor survival when compared to Groups A (no p53 or dm2

30 elevated levels) and B (elevated levels of only one of these proteins).

This is unexpected because, as discovered by the inventors, dm2 protein inactivates p53 transcriptional

35 activity, and therefore one might not have expected elevated

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levels of dm2 protein in a cell with elevated levels of p53 protein.

Group B (p53-/dm2+ and p53+/dm2-)

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Group B contains two subsets, normal levels of p53 with elevated levels of dm2 (p53-/dm2+) and elevated levels of p53 with normal levels of dm2 (p53+/dm2-). Of the three groups, Group B indicates a poorer prognosis than Group A,

10   but better prognosis than Group C.

subset p53+/dm2- of Group B:

The detection of elevated levels of p53, such as
15   mutated or overexpressed wild-type p53 genes and proteins, i.e. the p53+/dm2- group, indicate pre-cancer and cancer states. Mutant forms of p53 can inactivate the wild-type p53 function, and cells containing mutant p53 genes and proteins have an enhanced tumorigenic potential.
20   Furthermore, elevated levels of wild-type p53 protein may contribute to poor prognosis. DNA damage in cells or binding to viral oncogene products stabilize wild-type p53 protein and increase its concentration.

25   subset p53-/dm2+ of Group B:

The invention unexpectedly demonstrates that elevated levels of dm2 confer a similar property on cells, such as such as BALB/c 3T3 cells (3T3 DM cells) or pre-cancer or
30   cancer cells, as does elevated levels of p53. In both cases, the cells that express high levels of dm2 or p53 protein gain an enhanced tumorigenic potential in animals.

, The invention provides that, with regard to the p53-
35   /dm2+ group, despite the presence of normal levels of wild-

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type p53 allele or alleles in a cell, elevated levels of dm2
enhance the tumorigenic potential of the cell.

5   The invention provides a method of detecting a cancer
cell or cell at risk of becoming cancerous or pre-cancerous
wherein the cell contains two normal p53 alleles, i.e. wild-
type p53, or normal levels of p53, comprising determining
whether the level of dm2 in the biological sample is
abnormally elevated, meaning elevated in comparison with the
10   level of dm2 in normal biological samples. (see Figure 4).

Group A (p53-/dm2-)

15   Group A comprises normal levels of both p53 and dm2.
Precancer and cancer cells in this group are most likely due
to factors other than elevated levels of p53 and/or dm2. Of
the three groups, Group A indicates the best prognosis.

20

DETECTION OF ELEVATED LEVELS OF DM2 OR P53:

Amplification:

25   Amplification of the dm2 or p53 gene may be detected
using methods well known in the art, such as nucleic acid
probe technology. High copy numbers of DNA may, for
example, be detected using Southern blotting, and increased
amounts of RNA may be detected using Northern blotting (see
30   George, D.L. and Powers, V.E., Cell 24:117-123 (1981);
George, D.L. et al. EMBO J., 4:1199-1203 (1985); and Singh,
L. and Jones, K.W. Nucleic Acids Res., 12:5627-5638 (1984).

Overexpression:

35   Levels of dm2 and p53 protein in the biological sample

are detected by methods well known in the art, such as by
using anti-dm2 and anti-p53 antibodies and
immunohistochemical staining. A positive nuclear staining
when using immunohistochemistry indicate elevated levels of

5 p53 or dm2 or p53/dm2 complexes. Overexpressed p53 proteins
are associated with p53 gene mutations. In one embodiment
of the invention, dm2 and p53 nuclear overexpression in
tumors is classified into one of three groups by estimating
the percentage of tumor cell nuclei staining: (a) negative

10 (<20%), (b) heterogeneous (20-70%), (c) homogeneous (>70%).

In another embodiment of the invention, the elevated
level of dm2 is detected by a method indicating that a pre-
cancer or cancer state is 2-100 times that of normal

15 biological samples, such as cells. In a preferred
embodiment, the elevated level is a level 5-50 times that of
normal biological samples, such as cells.

Polyclonal and monoclonal antibodies may be prepared by

20 methods known in the art. Antibodies of this invention
include recombinant polyclonal or monoclonal Fab fragments
prepared in accordance with the method of Huse et al.,
Science 246:1275-1281 (1989). See Campbell, "Monoclonal
Antibody Technology, The Production and Characterization of

25 Rodent and Human Hybridomas" in Burdon et al., Eds,
Laboratory Techniques in Biochemistry and Molecular Biology,
Volume 13, Elsevier Science Publishers, Amsterdam (1985).
Methods for preparing polyclonal and monoclonal antibodies
that exhibit specificity toward single amino acid

30 differences between oncogenes are described by McCormick et
al. in U.S. Patent No. 4,798,787.

Briefly, polyclonal antibodies may be produced by
injecting a host mammal, such as a rabbit, mouse, rat, or

35 goat, with the p53 protein or a fragment thereof capable of

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producing antibodies that distinguish between mutant p53 and
wild-type p53. The peptide or peptide fragment injected may
contain the wild-type sequence or the mutant sequence. Sera
from the mammal are extracted and screened to obtain
5   polyclonal antibodies that are specific to the peptide or
peptide fragment. The same method may be applied to dm2
proteins.

In order to produce monoclonal antibodies, a host
10 mammal is inoculated with a peptide or peptide fragment as
described above, and then boosted. Spleens are collected
from inoculated mammals a few days after the final boost.
Cell suspensions from the spleens are fused with a tumor
cell in accordance with the general method described by
15 Kohler and Milstein in Nature 256, 495-497 (1975). In order
to be useful, a peptide fragment must contain sufficient
amino acid residues to define the epitope of the p53 or dm2
molecule being detected.

20   If the fragment is too short to be immunogenic, it may
be conjugated to a carrier molecule. Some suitable carrier
molecules include keyhole limpet hemocyanin and bovine serum
albumen. Conjugation may be carried out by methods known in
the art. One such method is to combine a cysteine residue
25 of the fragment with a cysteine residue on the carrier
molecule.

The peptide fragments may be synthesized by methods
known in the art. Some suitable methods are described by
30 Stuart and Young in "Solid Phase Peptide Synthesis," Second
Edition, Pierce Chemical Company (1984).

Suitable antibodies for the co-immunoprecipitation of
p53 and dm2 include PAb421 and Ab2. PAb421 recognizes the
35 carboxy-terminus of p53 from various species, including

15

human, mouse and rat p53, and is described by Harlow et al. in the Journal of Virology 39, 861-869 (1981). Ab2 is specific for the amino-terminus of human p53, and is available from Oncogene Science, Inc. of Manhassett, New

5 York. The dm2 protein does not immunoprecipitate when REF cells that do not express p53 are treated in the same way with the same antibodies.

The immunoprecipitates are recovered by centrifugation.
10 Following centrifugation or elution from the column, dm2 may be separated from the p53/dm2 complex by means of SDS PAGE. The single band at 90kD is cut and sequenced. This invention also provides an isolated dm2/p53 complex, obtained by the coimmunoprecipitating of dm2 with p53 from
15 a variety of transformed cells.

Another method for purifying dm2 is that generally described by Aebersold et al., Proc. Natl. Acad. Sci. USA 84, 6970-6974 (1987).
20

This invention provides hybridomas expressing monoclonal antibodies against the dm2 (p90) gene product. Certain of these hybridomas are deposited at the American Type Culture Collection (ATCC): 3G5 ATCC Accession No. HB
25 11182); 4B11 (ATCC Accession No. HB 11183); 2A10 (ATCC Accession No. HB 11184); 2A9 (ATCC Accession No. 11185); and 4B2 (ATCC Accession No. HB 11186). Hybridomas 3G5, 4B11, 2A10, 2A9 and 4B2, were deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on
30 the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession Nos. HB 11182, HB 11183, HB 11184, HB 11185 and HB 11186,
35 respectively.

ASSAYS FOR DETERMINING AND MODIFYING THE LEVEL OF Dm2 IN CELLS

The level of dm2 and/or p53 in cells is determined by
5 assays known in the art capable of recognizing amplified or
overexpressed dm2 and/or p53 genes or proteins.

A variety of assays are available for detecting
proteins with labeled antibodies. In a one-step assay, the
10 target molecule, if it is present, is immobilized and
incubated with a labeled antibody. The labeled antibody
binds to the immobilized target molecule. After washing to
remove unbound molecules, the sample is assayed for the
presence of the label.
15

In a two-step assay, immobilized target molecule is
incubated with an unlabeled antibody. The target molecule-
unlabeled antibody complex, if present, is then bound to a
second, labeled antibody that is specific for the unlabeled
20 antibody. The sample is washed and assayed for the presence
of the label, as described above.

Labeled anti-dm2 antibodies may be used to detect dm2
using imaging methods. One method for imaging comprises
25 contacting the tumor cell to be imaged with an anti-dm2
antibody labeled with a detectable marker. The method is
performed under conditions such that the labeled antibody
binds to the dm2. The antibody bound to the dm2 is
detected, thereby imaging and detecting the dm2.
30

The choice of marker used to label the antibodies will
vary depending upon the application. However, the choice of
marker is readily determinable to one skilled in the art.
These labeled antibodies may be used in immunoassays as well
35 as in histological applications to detect the presence of

17

tumors. The labeled antibodies may be polyclonal or monoclonal. In a preferred embodiment, the antibodies are monoclonal, and are the antibodies deposited with the ATCC listed above.

5

In preferred embodiments of the invention, the label may be a radioactive atom, an enzyme, or a chromophoric moiety. Some examples of radioactive atoms include P^{32}, I^{125}, H^3, and C^{14}. Some examples of enzymes include horseradish

10 peroxidase, alkaline phosphatase, beta-galactosidase, and glucose-6-phosphate dehydrogenase. Some examples of chromophoric moieties include fluorescein and rhodamine. The antibodies may be conjugated to these labels by methods known in the art. For example, enzymes and chromophoric

15 molecules may be conjugated to the antibodies by means of coupling agents, such as dialdehydes, carbodiimides, dimaleimides, and the like. Alternatively, conjugation may occur through a ligand-receptor pair. Some suitable ligand-receptor pairs include, for example, biotin-avidin or -

20 streptavidin, and antibody-antigen.

Antibodies against the dm2 protein may be used to detect elevated levels of the dm2 protein in cells or tissue. In one embodiment, antibodies that are directed at

25 epitopes of dm2 may be used in situ using immunohistopathology techniques. These methods have diagnostic use where there is a high risk of tissue becoming cancerous, such as in polyps or atypical breast tissue, or in tumor cells where there is a greater risk of metastasis

30 or recurrence.

The hybridomas 1F5, 6C10, 1D6, 4B2, 2E12, 3F3, 3G5, 3F8, 6H7, 2A9, 3G9, 1D11, 2A10, 1G2, 4B11 and 5B10, some of which were deposited with the ATCC (see above), produce

35 monoclonal antibodies against dm2 protein epitopes (see

18

Figure 1). The monoclonal antibodies react with both murine
and human protein epitopes, as well as with other species,
due to the homologous dm2 sequences conserved between the
species.

In another embodiment, cancer cells may release dm2 and
therefore increase the amount of dm2 in the blood or lymph
of a subject. Therefore, assays such as immunoassays may be
used to detect normal levels or levels of dm2 above normal
in cells or bodily fluids.

Screening for Therapeutics to Block dm2 Binding to Wild-Type
p53:

Assays may be used to screen for therapeutics to
inhibit dm2 binding to wild-type p53. The subject invention
discloses methods for selecting a therapeutic which forms a
complex with dm2 with sufficient affinity to prevent the
deleterious binding of dm2 to wild-type p53. The methods
include various assays, including competitive assays where
the dm2 is immobilized to a support, and is contacted with
both wild-type p53 and a labeled therapeutic either
simultaneously or in either consecutive order, and
determining whether the therapeutic effectively competes
with the wild-type p53 in a manner sufficient to prevent
binding of dm2 to wild-type p53. In another embodiment, the
wild-type p53 is labeled and the therapeutic is unlabeled.
In a further embodiment, the p53 is immobilized to a
support, and is contacted with both labeled dm2 and a
therapeutic (or unlabeled dm2 and a labeled therapeutic),
and determining whether the amount of dm2 bound to the p53
is reduced in comparison to the assay without the
therapeutic added. The dm2 may be labeled with the anti-dm2
antibodies of the subject invention.

19

In one embodiment, the method comprises:

a) contacting a solid support with a predetermined amount of
dm2 under conditions permitting dm2 to attach to the surface
5   of the support, such as by using an anti-dm2 antibody to
tether the dm2 to the solid support; b) removing any dm2
which is not bound to the support; c) contacting the solid
support to which the dm2 is bound with wild-type p53 under
conditions such that the wild-type p53 binds to the bound
10 dm2 and forms a complex therewith; d) removing any unbound
p53; e) contacting the dm2/p53 complex so formed with a
predetermined amount of the sample labeled with a detectable
marker under conditions such that the labeled potential
therapeutic present in the sample competes with the wild-
15 type p53 for binding to the bound dm2; f) quantitatively
determining   the   concentration   of   labeled   potential
therapeutic not bound to the solid support; and g) thereby
quantitatively determining the concentration of potential
therapeutic in the sample that specifically binds to dm2 to
20 block dm2 binding to wild-type p53.

The choice of solid support may be readily determined by one
skilled in the art. In one preferred method, the solid
support is a bead formed of an inert polymer, in another the
25 solid support is a microwell. The markers used in the
above-described method are a matter of choice to one skilled
in the art. It is preferred that the detectable marker is
an enzyme, a paramagnetic ion, biotin, a fluorophore, a
chromophore, a heavy metal, or a radioisotope. More
30 preferably, the marker is an enzyme, and most preferably,
the enzyme is horseradish peroxidase or alkaline
phosphatase.

A further embodiment of this method is wherein the potential
35 therapeutic is labeled with an enzyme and step (f) comprises

20

removing the labeled potential therapeutic which was not
bound to the solid support and contacting it with specific
substrate to the enzyme under conditions such that the
enzyme reacts with the substrate to form a detectable
5 product.

The subject invention also provides a method of
treating cancer in mammals including mice, rats, hamsters
and humans, which comprises blocking the deleterious binding
10 of dm2 to wild-type p53. One embodiment of this method
comprises blocking the binding of dm2 to wild-type p53 by
contacting the dm2 with a sufficient amount of anti-dm2
antibody. Another embodiment of this method comprises
blocking the binding of dm2 to wild-type p53 by contacting
15 the dm2 with an excess of an anti-idiotypic p53 antibody.
In another embodiment, dm2 anti-idiotypic antibodies may be
administered which do not block the transcription promoter
of p53. In another method for treating tumors, gene therapy
may be used to replace the amplified dm2 genes with a normal
20 number of dm2 genes to regulate elevated levels of p53. In
a further method of treating tumors, anti-sense gene therapy
is used to replace the amplified dm2 gene with an antisense
dm2 gene.

25 Methods for determining relative binding affinities may
be conducted by methods known in the art. For example, a
method for determining whether a p53 protein binds to hsc70
is described by Finlay et al.. in Mol. and Cell. Biol. 8,
531-539 (1988) and by Hinds et al.. in Mol. and Cell. Biol.,
30 7, 2863-2869 (1987). The method described in these papers
involves co-immunoprecipitation experiments with anti-p53
and anti-hsc70 antibodies.

35   **EXAMPLES**

21

Example 1

Isolation of cDNA clones.

A λgt11 cDNA library prepared from HeLa cells was
5   screened with the mouse mdm2 cDNA as probe under reduced
stringency.   The cDNA inserts were isolated from positive
phages and subcloned into the Bluescript vector for further
characterization.   A full length cDNA containing the entire
coding region was reconstructed from two overlapping clones
10 and completely sequenced using the method of Sanger (Sanger
et   al.   1977.   DNA   sequencing   with   chain-terminating
inhibitors. Proc. Natl. Acad. Sci. ·USA 14:5463-5467).

Generation of monoclonal antibodies against hdm2.

15   A cDNA clone obtained from the library screening
contained the N-terminal coding region of the hdm2 coding
region, truncated at the first methionine initiation codon.
This cDNA was recombined with the full length hdm2 cDNA to
obtain a coding region without leader sequence and the first
20 methionine. This coding sequence was then inserted into the
pQE11 vector (Quiagen) to obtain a complete open reading
frame with 6 histidine residues fused to the N-terminus of
hdm2.   The expression plasmid was then introduced into E.
coli, the Histidine-hdm2 fusion protein was purified by Ni-
25 $^{2+}$-NTA-agarose (Quiagen) column chromatography.   The major
protein species in the purified preparation has a mobility
similar to in vitro translated hdm2.

Balb/c mice were immunized with the E. coli produced
30 hdm2 protein.   Hybridomas were prepared using standard
procedures, and screened by enzyme-linked immunosorbent
assay and immunoprecipitation of in vitro translated hdm2
protein. Stable clones were established by three rounds of
cloning.

35

Isolation of human dm2 cDNA.

A λgt11 library constructed from HeLa cells was
screened using the mouse mdm2 cDNA under conditions of
reduced stringency.   A total of 14 positive clones were
5 isolated and the cDNA inserts subcloned into the Bluescript
vector for further analysis.   Preliminary restriction
mapping and partial sequencing showed that they represent
partial clones for the human dm2 cDNA (Fakharzadeh, S.S. et
al. 1991. Tumorigenic potential associated with enhanced
10 expression of a gene that is amplified in a mouse tumor cell
line.   EMBO J. 10:1565-1569).   A full length coding region
was constructed from two overlapping cDNA clones and
sequenced.   The DNA sequence of this cDNA clone, designated
hdm2, is similar to the published hdm2 sequence (Oliner,
15 J.D. et al. 1992. Amplification of a gene encoding a p53-
associated protein in human sarcomas. Nature 358:80-83),
with complete identity within the coding region and a few
differences in the noncoding regions.   The fact that these
two cDNA clones were obtained from two very different
20 sources (HeLa cell vs. colon carcinoma), yet have identical
coding sequences, suggests that they may represent the wild-
type hdm2 coding sequence or a systematic mutation is
present in different cancer cells.

25 **Example 2**

A cohort of 211 soft tissue sarcomas of adults that were
clinically and pathologically well characterized were
analyzed in this example.   In a subgroup of 73 patients,
30 tumor and normal tissue specimens were available.   This
group of 73 patients was used to address the molecular and
biological considerations of the study.   However,
clinicopathological correlations were conducted using
immunohistochemistry and information regarding 211 patients
35 with soft tissue sarcomas obtained from a frequently updated

23

database of clinical and pathological information available for patients at Memorial Sloan-Kettering Cancer Center.

TISSUE.

5   Of the cohort of 211 adult patients affected with soft tissue sarcomas (STS) used for this example, the tumor lesions analyzed included 71 liposarcomas, 53 leiomyosarcomas, 22 malignant fibrous histiocytoma, 15 fibrosarcomas, 15 peripheral nerve sheath tumors (PNST), 13

10   synovial sarcomas, 4 rhabdomyosarcomas, and 18 undifferentiated sarcomas. The majority of STS analyzed presented as primary tumors (n=129), while the remaining lesions were either recurrent (n=39) or metastatic (n=41). Presentation status in two cases was unknown. Of the 211

15   STS analyzed, 169 cases were classified as high grade sarcomas, while 39 tumors were considered to be low grade lesions. The grade of 3 cases was unknown. The median and mean follow-up times for this cohort of patients were 29 and 34 months, respectively. Complete follow-up data was

20   available for 209 of the 211 patients.

        Tumor specimens were embedded in cryopreservative solution (OCT compound, Miles Laboratories, Elkhart, IN), snap frozen in isopentane and stored at -70°C.

25   Representative hematoxylin-eosin stained sections of each block were examined microscopically to confirm the presence of tumor, as well as to evaluate percentage of tumor cells comprising these lesions and extent of tumor necrosis. Adjacent tumor and normal tissue specimens were also

30   collected for molecular genetic assays in 73 of 211 cases (see below). These tissue samples were immediately frozen after surgical removal and stored at -70°C prior to DNA extraction.

35

MONOCLONAL ANTIBODIES AND IMMUNOHISTOCHEMISTRY.

A panel of mouse monoclonal antibodies to the p90 gene
encoded gene product were used for the present study.
5   Antibody 4B2 detects an epitope located in the amino-
terminal region.   Antibodies 2A9 and 2A10 identify two
distinct epitopes in the central portion of p90. Antibody
4B11 recognizes a sequence located in the carboxy-terminal
region of p90.   Three mouse monoclonal antibodies detecting
10 different epitopes on p53 proteins were used for the present
study.   Anti-p53 antibody PAb1801 (Ab-2, Oncogene Science,
Manhasset, New York) recognizes an epitope located between
amino acids (aa) 32 to 79 of both wild-type and mutant human
p53 proteins (Banks, L. et al.   (1986) *Eur J Biochem* **159**,
15 529-534).   Antibody PAb240 (Ab-3, Oncogene Science)
recognizes a conformational epitope located between aa 156
to 335 characteristic of certain mutant p53 products
(Gannon, J. V. et al. (1990) *EMBO J* **9**, 1595-1602). Antibody
PAb1620 (Ab-5, Oncogene Science) reacts specifically with
20 wild type p53 (Ball, R. K. et al. (1984) *EMBO J* **3**,
1485-1491).   MIgS-Kp I, a mouse monoclonal antibody of the
same subclass as the anti-p90 and anti-p53 antibodies, was
used as a negative control at similar working dilutions.

25   The avidin-biotin peroxidase method was performed on 5
um thick frozen tissue sections fixed with cold methanol-
acetone (1:1 dilution).   Briefly, sections were incubated
for 15 minutes with 10% normal horse serum (Organon Tecknika
Corp., Westchester, PA), followed by a two hour incubation
30 with appropriately diluted primary antibodies (2A9, 4B2 and
4B11 were used at 1:100 dilution, while 2A10 was used at
1:1000 dilution)(Ab-2 was used at 200 ng/ml; Ab-3 at 250
ng/ml and Ab-5 at 3 ug/ml).   After extensive washing,
sections were subsequently incubated for 30 minutes with
35 biotinylated horse anti-mouse IgG antibodies at 1:200

25

dilution (Vector Laboratories, Burlingame, CA) and avidin-
biotin peroxidase complexes (Vector Laboratories at 1:25
dilution for 30 minutes). Diaminobenzidine (0.06% DAB) was
used as the final chromogen and hematoxylin as the nuclear
5   counterstain.

Immunohistochemical evaluation was done by at least two
independent investigators, scoring the estimated percentage
of tumor cells that showed nuclear staining. Both p90 and
10   p53 nuclear immunoreactivities were classified into three
categories defined as follows: negative (<20% tumor cells
displaying nuclear staining), heterogeneous (20-79% tumors
cells with nuclear reactivities), and homogeneous (> 80%
tumor cells with intense nuclear staining).
15

SOUTHERN BLOTTING AND RFLP ANALYSES.

A human dm2 cDNA fragment probe of 1.6 kb, pHDM
(EcoRI), was used in Southern blots to assess gene
20   amplification. A b-actin probe,   (EcoRI), was used as a
control. Two probes were used for the analysis of allelic
deletions of the short arm of chromosome 17, PYNZ22
(17p13.3, D17S5, TaqI) and php53B (17p13.1, p53, BglII).
Southern analysis was performed as described (Presti, J. C.
25   et al. (1991) Cancer Res 51, 5405; Dalbagni, G. et al.
(1993) Diagnostic Molecular Pathology 2, 4-13). Briefly,
DNA was extracted by the non-organic method developed by
Oncor (Oncor, Gaithersburg, MD) from paired normal and tumor
samples, digested with the appropriate restriction enzymes,
30   electrophoresed in 0.7% agarose gel, and blotted onto nylon
membranes. The membranes were prehybridized with Hybrisol
I (Oncor) at 42°C for one hour, and hybridized with probes
labelled to high specific activity with [P32] dCTP
overnight. Membranes were then washed and subjected to
35   autoradiography using intensifying screens at -70°C.

26

Densitometry using an Ultrascan XL Laser Densitometer
(Pharmacia LKB Biotechnology, Piscataway, NJ), as well as a
Betascope 630 Blot Analyzer (Betagen, Waltham, MA), was
performed to confirm the results. A case was considered to
5   have a dm2 amplification when it had at least 5 copies
gene/cell. Loss of heterozygosity (LOH) was defined as a
greater than 40% decrease in signal intensity of an allele
in the tumor samples (Presti, J. C. et al. (1991) *Cancer Res*
51, 5405; Olumi, A. F. et al. (1990) *Cancer Res* **50**,
10 7081-7083).

SINGLE STRANDED CONFORMATIONAL POLYMORPHISM (SSCP) ANALYSIS
AND DNA SEQUENCING.

15   These studies were performed according to a slight
modification of the method reported by Orita et al (Orita,
M. et al. (1989) *Genomics* **5**, 874-879). Amplifications were
performed using 100 ng of genomic DNA extracted from the
samples described above. The primers used were obtained
20 from intronic sequences flanking exons 5 through 9 of the
human p53 gene, sequences being previously published (Moll,
U. M. et al. (1992) *Proc Natl Acad Sci USA* **89**, 7262-7266).
DNA was amplified following 30 cycles of PCR (30s at 94°C,
30s at 58°C for exons 8 and 9 and 63°C for exons 5, 6 and 7,
25 and finally 60s for all samples at 72°C) using a Thermal
Cycler (Perkin Elmer Cetus). Amplified samples were then
denatured and loaded onto a non-denaturing acrylamide gel
containing 10% glycerol and run at room temperature for 12-
16 hours at 10-12 watts. Gels were dried at 80°C under
30 vacuum and exposed to X-ray film at -70°C for 4-16 hours.

Amplification of genomic DNA for sequencing assays was
independent of that used for SSCP analysis, using 35 cycles
(60s at 94°C, 60s at 58°C and 63°C - as above, and 90s at
35 72°C). DNA fragments were isolated from 2% low melting point

27

agarose gels, purified and sequenced by the dideoxy method
(Sanger, F. et al. (1977) *Proc Natl Acad Sci USA* **74**,
5463-5467). Both strands were sequenced for each DNA
analyzed, and genomic DNA from control samples containing
5   wild-type p53 were sequenced in parallel to confirm the
mutations.

DM2 AMPLIFICATION AND OVER-PRODUCTION OF DM2 PROTEINS.

10

        Amplification of the dm2 gene was detected in 11 of 73
adult soft tissue sarcomas (STS), ranging from 5- to 35-
fold. Dm2 amplifications were more frequently detected in
high grade (7 cases) than in low grade (4 cases) STS.
15   Amplifications were more commonly observed in metastatic (3
of 11 cases, 27%) than in primary sarcomas (4 of 48 cases,
8%).

        The pattern of immunostaining of anti-dm2 antibodies
20   was first assessed using 3T3-Balbc and 3T3-DM cells. A
strong nuclear staining was seen in DM cells, reported to
have an amplified dm2 gene and to overexpress dm2 proteins;
while Balb-c cells were unreactive (Figure 4) and have very
low levels of dm2 proteins. Six of the 11 amplified cases
25   showed over 20% tumor cells displaying nuclear
immunoreactivities with anti-dm2 antibodies. However, the
remaining 5 cases were unreactive. Seventeen of the 62
cases with an apparent non-amplified dm2 gene showed
elevated levels of dm2 proteins as detected by the dm2
30   antibodies using tissue sections (dm2-positive phenotype).

p53 DELETIONS, POINT MUTATIONS, AND p53 NUCLEAR
IMMUNOREACTIVITIES.

35   73 pairs of somatic and tumor DNA were examined with

28

two different probes for the short arm of chromosome 17. Deletions of the short arm of chromosome 17 were found in 27 of 51 (53%) informative cases examined. Loss of heterozygosity (LOH) of chromosome 17p was observed in both
5 low and high grade sarcomas. Chromosome 17p LOH was more frequently found in metastatic (6 of 8 cases, 75%) than in primary (13 of 33 cases, 41%) tumors.

       To further characterize the specific intragenic
10 mutations of p53 as they may relate to p53 overexpression in these cells, 73 STS were analyzed using SSCP (exons 5 through 9) and those positive for that assay were followed by DNA sequencing. Confirmation of the presence of a mutation was revealed in 14 cases. Eleven of these 14 STS
15 displayed p53 nuclear immunoreactivities for antibody PAb1801. Point mutations were characterized by sequencing in 7 of these 11 sarcomas, 5 showed AT to GC transitions, while 2 were GC to AT transitions. In 4 cases shifts in mobility were detected by SSCP, but no sequencing was
20 conducted to identify the mutation. Three of the 14 mutant cases showed negative immunostaining results for PAb1801. One mutation was identified in codon 165, producing a stop codon. Another case had a C deletion at codon 278, producing a stop codon at position 344. The other mutation
25 occurred in exon 5 affecting a splice donor site. All informative mutants for chromosome 17p status but one had a concomitant deletion of the short arm. In addition, 13 cases showed a positive nuclear staining signal without evidence of point mutations for the exons under study.
30

       Overall, 56 of the 211 STS analyzed displayed a positive nuclear pattern of immunostaining for PAb1801 (Table 1). There was a significant difference between p53-positive phenotype and tumor grade. Moreover, patients
35 affected with STS that showed p53 nuclear immunoreactivities

29

in over 20% tumor cells had significantly reduced survival rates.

TABLE 1

Soft Tissue Sarcomas
High Expression of p53 and mdm-2

	p53 over-expression	p53 normal	totals
mdm-2 over-expression	22	54	76/211 (36%)
mdm-2 normal	34	101	135/211
Totals	56/211 (26.5%)	155/211	

ALTERED GENOTYPE AND PHENOTYPE OF DM2 AND P53:
CLINICOPATHOLOGICAL IMPLICATIONS.

5   Only one of the subgroup of 73 cases exhibited an
amplified dm2 and a mutant p53 gene, and this was a
metastatic fibrosarcoma. However, 22 of a total cohort of
211 cases showed positive nuclear immunoreactivities for
both dm2 and p53 proteins in consecutive tissue sections
10 (Table 1). The pattern of staining of these molecules, in
cases on which they were co-expressed, was in general
heterogeneous. When comparing the combined phenotypes
(Group A: dm2-/p53-; Group B: dm2+/p53- and dm2-/p53+; and
Group C: dm2+/p53+) versus clinicopathological parameters,
15 a correlation was observed between the positive phenotype
and variables for a poor prognosis. The data support the
conclusion that Group A correlates with the best prognosis,
and Group C correlates with the poorest prognosis (see
Figure 3).
20

Example 3

        p90 may be used to prepare antibodies that are capable
25 of immunoprecipitating p90 or co-immunoprecipitating p90
with p53. p90 may be isolated from the precipitate and
purified. The anti-p90 antibodies may be polyclonal or
monoclonal.

30   One method for preparing polyclonal antibodies to p90
is as follows:

Procedure for p90 antibody production in rabbits:

35   The p90 protein fragment is at a concentration of 0.7

32

mg/ml. Immunizations are given as follows:

Day 0   25 µg in 200 µl PBS plus 200 µl RIBI (adjuvant)
Day 7   50 µg in 200 µl PBS plus 200 µl RIBI (adjuvant)
5   Day 14   50 µg in 200 µl PBS plus 200 µl RIBI (adjuvant)
Day 21   REST
Day 28   50 µg in 200 µl PBS plus 200 µl RIBI
Day 39   Bleed -- assay
Day 52   Boost animal with 50 µg in 200 µl PBS plus 200 µl
10   RIBI
Day 59   Bleed ---- 7 days after last injection assay
Day 62   Exsanguinate

Bleeds were assayed by ELISA, coating wells at 200
15   ng/well overnight at 4°C. Block 2% BSA for 1 hr. at 37°C.
Make serum dilutions in 1% BSA and incubate 2 hrs. at 37°C.
Secondary antibody dilutions (TAGO Goat anti-rabbit
peroxidase -- cat.# 6430) in 1% BSA and incubate 37°C for 1
hr. Sera titered out to 1:25000 (cutoff absorbance 0.400 at
20   450 nm) for the first assay and >50000 for the final assay.
Develop with Kirkegaard & Perry TMB Peroxidase Substrate
Solution(s) and read absorbance at 450 nm.

Example 4

25

The hybridomas 1F5, 6C10, 1D6, 4B2, 2E12, 3F3, 3G5,
3F8, 6H7, 2A9, 3G9, 1D11, 2A10, 1G2, 4B11 and 5B10 deposited
with the ATCC (see above) produce monoclonal antibodies
against p90 protein epitopes (see Figure 1). The following
30   protocols use these monoclonal antibodies and other anti-p90
antibodies as primary antibodies for detecting the DM2
gene-coded product, the p90 protein, by immunohistochemical
methods in tissues, preferably human tissues.

35   The avidin-biotin immunoperoxidase technique is

33

suitable due to the high sensitivity that it renders. Sections of human normal and tumor tissues are cut using a cryostat and placed on microslides. These sections are then incubated with blocking serum, followed by hydrogen peroxide

5   and avidin-biotin blocking. Primary antibodies, i.e. anti-p90 antibodies, are then used at an appropriate concentration. The appropriate concentration is empirically determined for each antibody by performing titrations. Sections are then incubated with biotinylated secondary

10 horse anti-mouse antibodies, followed by avidin-biotin peroxidase complexes. The final reaction is developed using diaminobenzidine. Sections are then counterstained with hematoxylin and mounted with permount for final analysis.

15   Using such immunohistochemical methods, typically only elevated levels of the p90 protein, not normal levels, are detectable in the tissues since, for those tissues examined in these cases, the monoclonal antibodies can detect only elevated levels of p90 protein in cells.

20

IMMUNOHISTOCHEMISTRY
AVIDIN-BIOTIN-PEROXIDASE METHOD
PARAFFIN EMBEDDED TISSUE SECTIONS

25 1)   Place 5 μm tissue sections on poly-L-lysine coated slides.

    2)   Place sections in 60C oven for 30 minutes to melt paraffin.

30

    3)   Cool slides at room temperature then process for deparaffinization and rehydration:
             xylene - 3 times (5 minutes each)
             100% ethanol - 3 times (3 minutes each)
35   95% ethanol - 3 times (3 minutes each)

34

4)   Wash in distilled H_2O and transfer to PBS.

5)   Quench with 1% H_2O_2 for 15 minutes to eliminate endogenous peroxidase activity.

6)   Wash in PBS - 3 times.

7)   In tissues that contain biotin (such as liver, kidney, brain, etc.), apply Avidin-Biotin blocking kit (Vector) in order to eliminate endogenous biotin activity. Solutions should be applied subsequently (Avidin, then biotin) and slides should be incubated for 15 minutes with each solution.

8)   Wash in PBS.

9)   Enzyme digestion - enzyme selection determined empirically as optimally for each antibody.

Common enzymes:

Pepsin (Porcine Stomach Mucosa, Sigma): HCL (250 ml distilled H_2O + 200 µl HCl) + 0.25 grams pepsin ---> incubation 30 minutes.

Trypsin (Bovine Pancreas Type I, Sigma): 250 ml TRIS + 0.0625 grams trypsin ---> incubate for 5 minutes; wash in distilled H_2O, then incubate for 15 minutes with trypsin inhibitor (Sigma): 250 ml PBS + 0.025 gms trypsin inhibitor.

Pronase (Calbiochem Behring): 250 ml TRIS + 0.0055 gms pronase ---> incubate for 4 minutes;

Ficin (suspension - Sigma) - ready to use, dropwise, incubation 45 minutes.

35

Saponin (detergent - Sigma): 250 ml distilled H_2O + 0.125 gms Saponin ---> incubation 30 minutes.

10)   Wash slides in distilled H_2O and transfer to PBS.

11)   Apply blocking serum - 10% normal serum (species specific - same species as the secondary antibody) - incubation 20-30 minutes.

12)   Vacuum suction off the blocking serum and apply appropriately diluted primary antibody - incubation overnight at 4°C in a humid chamber. The primary antibody is selected from the anti-p90 monoclonal antibodies produced by the following hybridomas: 1F5, 6C10, 1D6, 4B2, 2E12, 3F3, 3G5, 3F8, 6H7, 2A9, 3G9, 1D11, 2A10, 1G2, 4B11 and 5B10. The appropriate dilution of the primary antibody is an empirically determined optimal concentration.

13)   Extensive washing with PBS ---> 3 changes (5 minutes each)

14)   Apply appropriately diluted biotinylated secondary antibody --> 30 minutes incubation.

15)   Wash with PBS (3 x 5 minutes).

16)   Avidin-biotin complex (Vector) diluted 1:25 (equal ratio A B) ---> incubation 30 minutes.

17)   Wash with PBS (3 x 5 minutes).

18)   Substrate chromogen solution:
      peroxidase-diaminobenzadine (0.06% DAB)
      (5 mg DAB/ 100 ml PBS + 100 ul 0.3% H_2O_2)

Incubate until desired color intensity has developed
(approximately 5 minutes)

19)   Hematoxylin counterstain.

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IMMUNOHISTOCHEMISTRY
AVIDIN-BIOTIN-PEROXIDASE METHOD - FROZEN TISSUE SECTIONS

10 1.   5 μm frozen tissue sections - leave at room temperature
         for at least 30 minutes to thaw and dry sections.

    2.   Apply proper fixative for 10 minutes (fixative
         optimally chosen for each antibody).
15

    3.   Quench with 0.1% H_2O_2 for 15 minutes to eliminate
         endogenous peroxidase activity.

    4.   Wash in PBS - 3x.
20

    5.   Avidin-biotin blocking kit in tissues rich with
         endogenous biotin. Avidin - incubate 15 minutes, wash
         with PBS and then biotin -15 minutes.

25 6.   Wash in PBS - 3x.

    7.   Apply blocking serum - 10% normal serum (species
         specific, same species as the secondary antibody) 10-30
         minutes incubation in humid chamber.
30

    8.   Suction off the blocking serum and apply appropriately
         diluted primary antibody incubation 1-2 hours. The
         primary antibody is selected from the anti-p90
         monoclonal antibodies produced by the following
35   hybridomas: 1F5, 6C10, 1D6, 4B2, 2E12, 3F3, 3G5, 3F8,

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6H7, 2A9, 3G9, 1D11, 2A10, 1G2, 4B11 and 5B10. The appropriate dilution of the primary antibody is an empirically determined optimal concentration, typically 1-1000 volume/volume of hybridoma supernatant/phosphate

5   buffered saline (PBS).

9.   Wash extensively in PBS.

10.   Apply appropriately diluted biotinylated secondary
10   antibody -30 minutes incubation.

11.   Wash with PBS - 3x.

15   12.   Avidin-biotin complex (Vector) dilution 1:25 (equal
          ratio A:B) - 30 minutes.

13.   Wash PBS and PBS/Triton - 3x.

20   14.   S u b s t r a t e   c h r o m a g e n   s o l u t i o n :
          peroxidase-diaminobenzidine (0.06% DAB) approximately
          5 minutes.

15.   Hematoxylin counterstain.

CLAIMS

We claim:

1.   A method of diagnosing cancer by determining the level of p53 and dm2 in a biological sample, whereby an elevated level of either p53 or dm2 or both p53 and dm2 indicates a cancer diagnosis.

2.   A method of predicting the progress of cancer by determining the level of p53 and dm2 in a biological sample, whereby an elevated level of either p53 or dm2 or both p53 and dm2 indicates a poor prognosis.

3.   A method of classifying a biological sample into one of three groups, the method comprising determining whether the level of either p53 or dm2 in the sample is abnormally elevated, whereby the first group comprises no abnormal elevation of either the level of p53 or dm2, the second group comprises abnormal elevation of the level of p53 and no abnormal elevation of the level of dm2 or abnormal elevation of the level of dm2 and no abnormal elevation of the level of p53, and the third group comprises abnormal elevation of the level of both p53 and dm2.

4.   A method of assessing a subject's prognosis by obtaining a biological sample from the subject, classifying the sample's group using the method of claim 3, whereby, of the three groups, the third group indicates the worst prognosis.

5.   A method of assessing a subject's prognosis by obtaining a biological sample from the subject, determining the sample's group using the method of claim 3, whereby, of the three groups, the first group indicates the best

prognosis.

6.   A method according to claim 3, wherein the level
of dm2 gene amplification or expression is determined by
using probes.

7.   A method according to claim 6, wherein the probes
are antibodies.

8.   A method according to claim 7, wherein the
antibodies are monoclonal.

9.   A method according to claim 8, wherein the
antibodies are selected from the group consisting of claims
14-18.

10.   A monoclonal antibody of claim 27, 28, 29, 30 or
31 bound to a toxin.

11.   A method of treating a patient with a tumor by
contacting the tumor with an antibody of claim 10 under
conditions such that the antibody binds to the tumor.

12.   A monoclonal antibody of claim 27, 28, 29, 30 or
31 labeled with a detectable marker.

13.   A method for imaging tumors which comprises
contacting the tumor to be imaged with an anti-dm2 antibody
of claim 12, under conditions such that the antibody binds
to the tumor and detecting the antibody bound thereto,
thereby imaging the tumor.

14.   A method of detecting in a biological sample
cancer cells or cells at risk of becoming cancerous or pre-
cancerous, wherein the cells contain at least one normal p53

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allele, the method comprising determining whether the level of dm2 in the biological sample is abnormally elevated in comparison with the level of dm2 expression, whereby an abnormally elevated level of dm2 in the biological sample indicates cancer cells or cells at risk of becoming cancerous or pre-cancerous.

15. A method according to claim 14, wherein the elevated level is determined by immunohistochemical staining of the biological sample.

16. A method according to claim 14, wherein the level of dm2 gene amplification or expression is determined by using probes.

17. A method according to claim 16, wherein the probes are nucleic acid probes.

18. A method according to claim 16, wherein the probes are antibodies.

19. A method according to claim 18, wherein the antibodies are monoclonal.

20. A p53/dm2 complex.

21. A method according to claim 19, wherein the monoclonal antibody is produced by hybridoma 3G5, deposited under ATCC Accession No. HB 11182.

22. A method according to claim 19, wherein the monoclonal antibody is produced by hybridoma 4B11, deposited under ATCC Accession No. HB 11183.

23. A method according to claim 19, wherein the

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monoclonal antibody is produced by hybridoma 2A10, deposited under ATCC Accession No. HB 11184.

24. A method according to claim 19, wherein the monoclonal antibody is produced by hybridoma 2A9, deposited under ATCC Accession No. HB 11185.

25. A method according to claim 19, wherein the monoclonal antibody is produced by hybridoma 4B2, deposited under ATCC Accession No. HB 11186.

26. A method according to claim 19, wherein the monoclonal antibody recognizes the same epitope or epitopes as those recognized by a monoclonal antibody produced by a hybridoma selected from the group consisting of hybridoma 3G5, deposited under ATCC Accession No. HB 11182; hybridoma 4B11, deposited under ATCC Accession No. HB 11183; hybridoma 2A10, deposited under ATCC Accession No. HB 11184; hybridoma 2A9, deposited under ATCC Accession No. HB 11185; and hybridoma 4B2, deposited under ATCC Accession No. HB 11186.

27. A method according to claim 19, wherein the monoclonal antibody competes with the same epitope or epitopes as those recognized by a monoclonal antibody produced by a hybridoma selected from the group consisting of hybridoma 3G5, deposited under ATCC Accession No. HB 11182; hybridoma 4B11, deposited under ATCC Accession No. HB 11183; hybridoma 2A10, deposited under ATCC Accession No. HB 11184; hybridoma 2A9, deposited under ATCC Accession No. HB 11185; and hybridoma 4B2, deposited under ATCC Accession No. HB 11186.

28. A method according to claim 27, wherein the competing monoclonal antibody blocks between 50-100% of the epitope or epitopes recognized by the monoclonal antibody produced by

a hybridoma selected from the group consisting of hybridoma
3G5, deposited under ATCC Accession No. HB 11182; hybridoma
4B11, deposited under ATCC Accession No. HB 11183; hybridoma
2A10, deposited under ATCC Accession No. HB 11184; hybridoma
2A9, deposited under ATCC Accession No. HB 11185; and
hybridoma 4B2, deposited under ATCC Accession No. HB 11186.

29.   The monoclonal antibody produced by hybridoma 3G5,
deposited under ATCC Accession No. HB 11182.

30.   The monoclonal antibody produced by hybridoma
4B11, deposited under ATCC Accession No. HB 11183.

31.   The monoclonal antibody produced by hybridoma
2A10, deposited under ATCC Accession No. HB 11184.

32.   The monoclonal antibody produced by hybridoma 2A9,
deposited under ATCC Accession No. HB 11185.

33.   The monoclonal antibody produced by hybridoma 4B2,
deposited under ATCC Accession No. HB 11186.

34.   A monoclonal antibody that recognizes the same
epitope or epitopes as those recognized by a monoclonal
antibody produced by a hybridoma selected from the group
consisting of hybridoma 3G5, deposited under ATCC Accession
No. HB 11182; hybridoma 4B11, deposited under ATCC Accession
No. HB 11183; hybridoma 2A10, deposited under ATCC Accession
No. HB 11184; hybridoma 2A9, deposited under ATCC Accession
No. HB 11185; and hybridoma 4B2, deposited under ATCC
Accession No. HB 11186.

35.   A monoclonal antibody that competes with the same
epitope or epitopes as those recognized by a monoclonal
antibody produced by a hybridoma selected from the group

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consisting of hybridoma 3G5, deposited under ATCC Accession
No. HB 11182; hybridoma 4B11, deposited under ATCC Accession
No. HB 11183; hybridoma 2A10, deposited under ATCC Accession
No. HB 11184; hybridoma 2A9, deposited under ATCC Accession
No. HB 11185; and hybridoma 4B2, deposited under ATCC
Accession No. HB 11186.

    36.   A monoclonal antibody according to claim 35 that
blocks between 50-100% of the epitope or epitopes recognized
by the monoclonal antibody produced by a hybridoma selected
from the group consisting of hybridoma 3G5, deposited under
ATCC Accession No. HB 11182; hybridoma 4B11, deposited under
ATCC Accession No. HB 11183; hybridoma 2A10, deposited under
ATCC Accession No. HB 11184; hybridoma 2A9, deposited under
ATCC Accession No. HB 11185; and hybridoma 4B2, deposited
under ATCC Accession No. HB 11186.

    37.   A hybridoma selected from the group consisting of
hybridoma 3G5, deposited under ATCC Accession No. HB 11182;
hybridoma 4B11, deposited under ATCC Accession No. HB 11183;
hybridoma 2A10, deposited under ATCC Accession No. HB 11184;
hybridoma 2A9, deposited under ATCC Accession No. HB 11185;
and hybridoma 4B2, deposited under ATCC Accession No. HB
11186.

    38.   Any epitope recognized by a monoclonal antibody
selected from the group of monoclonal antibodies of claims
29-33.

    39.   An epitope recognized by a monoclonal antibody of
claim 31.

    40.   A diagnostic kit comprising:
        (a)   a container comprising an anti-p53 antibody
              that recognizes a p53 protein; and

(b) a container comprising an anti-dm2 antibody
     that recognizes a dm2 protein.

41. A diagnostic kit of claim 40, wherein the
antibodies are labeled.

42. A diagnostic kit of claim 40, further comprising:
     (a) a labeled antibody that recognizes the anti-
          p53 antibody; and
     (b) a labeled antibody that recognizes the anti-
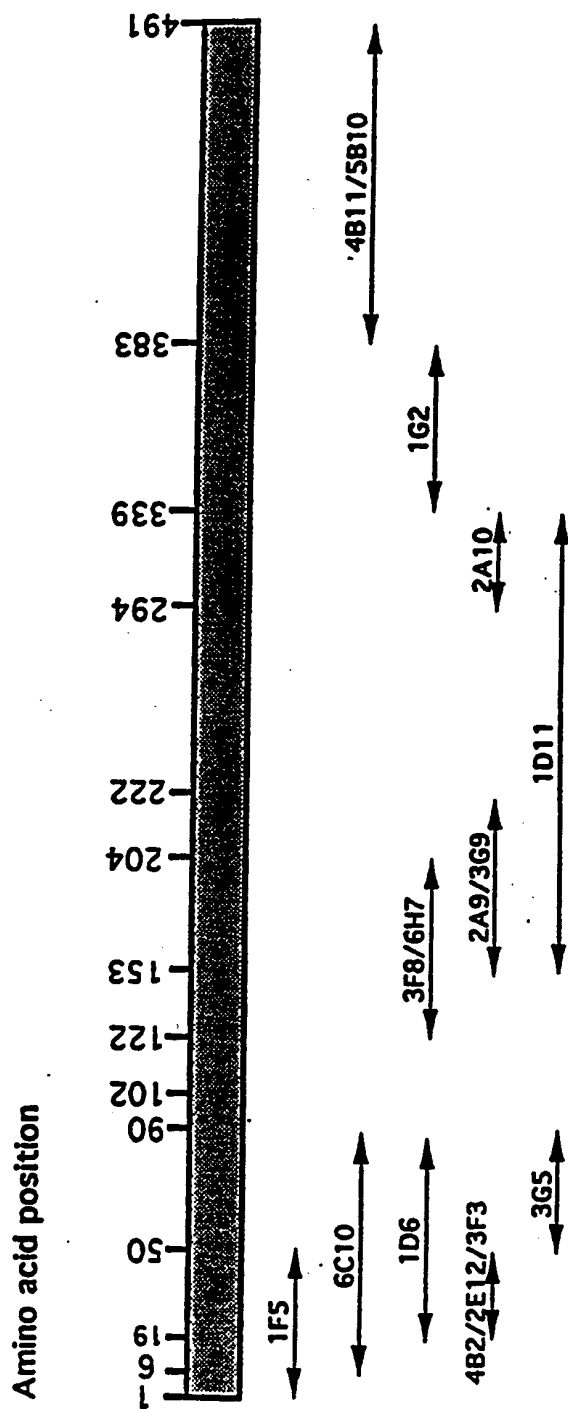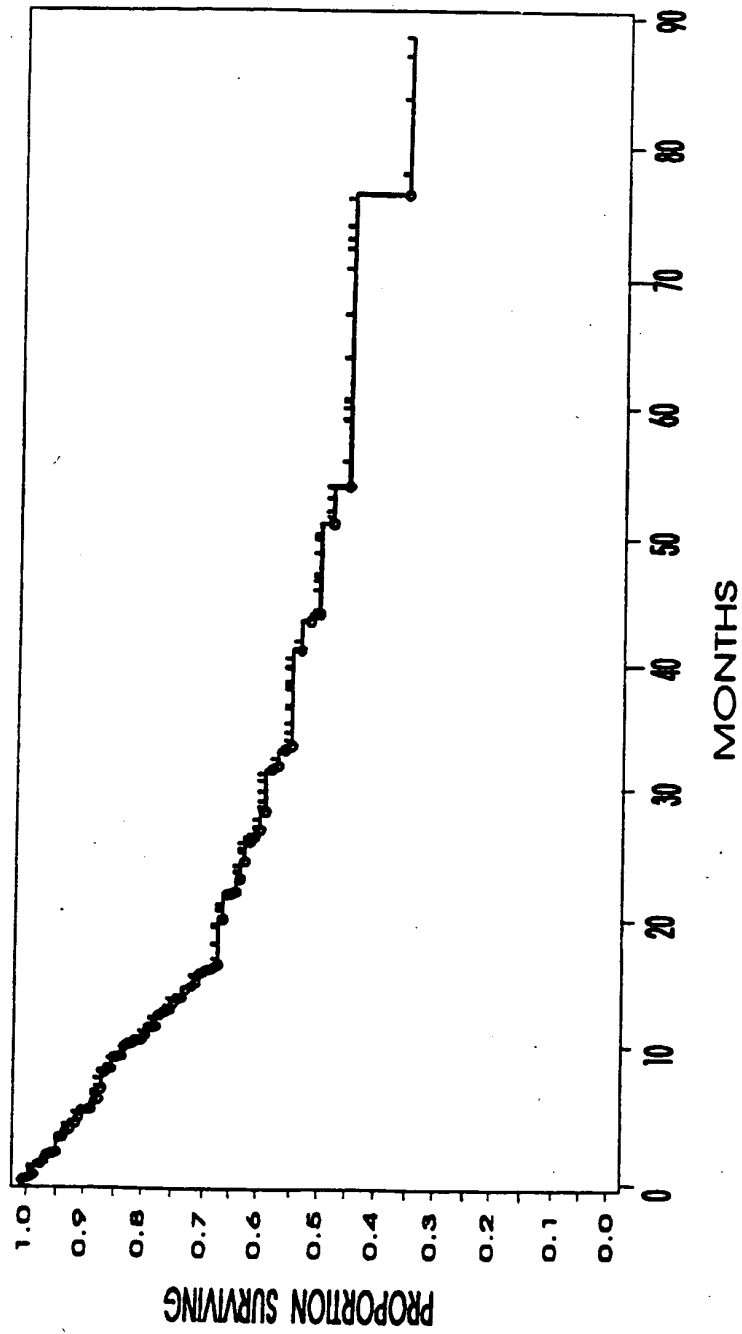   dm2 antibody.
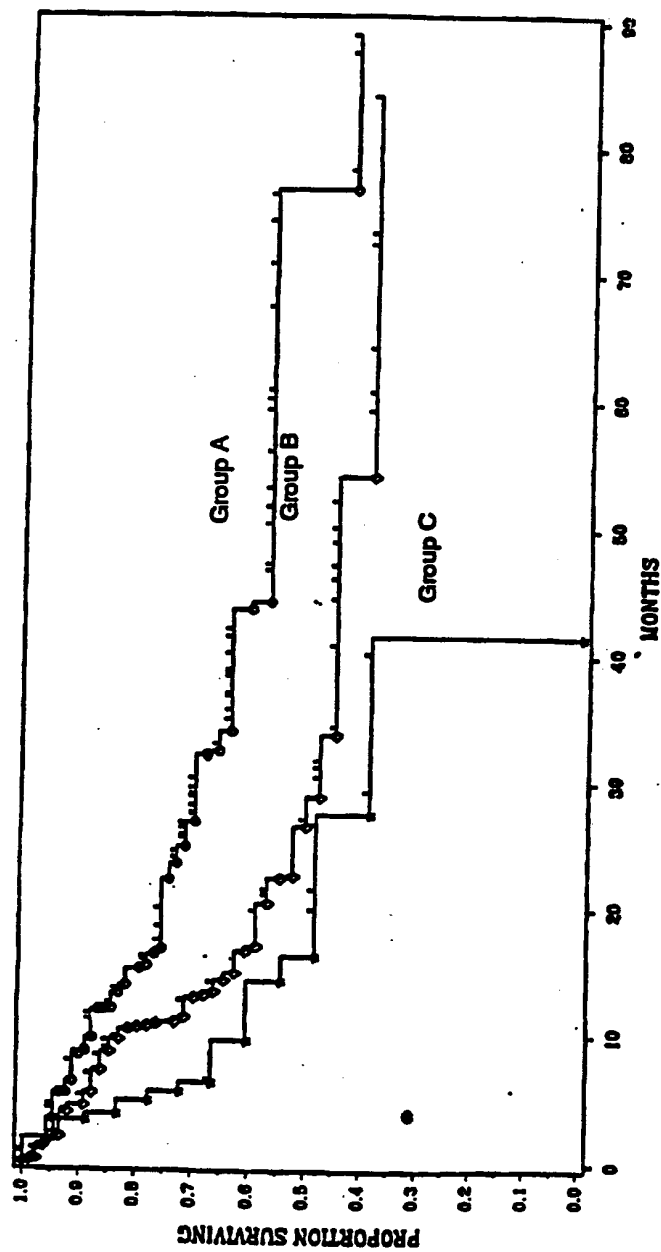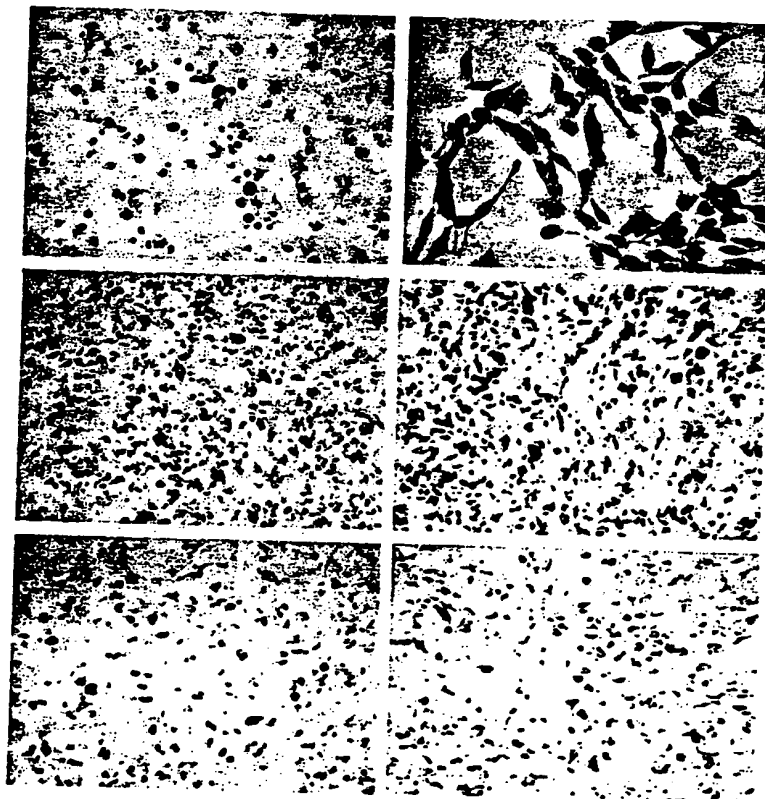
FIGURE 1

FIGURE 2

SUBSTITUTE SHEET

FIGURE 3

FIGURE 4

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SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; G01N 33/574; C12N 15/02; A61K 39/395; C07K 13/00, 15/28
US CL :435/6, 7.23, 172.2; 514/2; 530/350, 388.8

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/6, 7.23, 172.2; 514/2; 530/350, 388.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Biosis

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Lancet, Vol. 335, Issued 24 March 1990, Iggo et al., "Increased Expression of Mutant Forms of p53 Oncogene in Primary Lung Cancer", pages 675-679, see entire document.	1-9, 13-19, 40-42
Y	EMBO J., Vol. 10, Number 6, Issued 1991, Fakharzadeh et al., "Tumorigenic Potential Associated With Enhanced Expression of a Gene that is Amplified in a Mouse Tumor Cell Line", pages 1565-1569, see entire document.	1-9, 13-19, 40-42
Y	US, A, 4,762,706 (McCormick et al.) 09 August 1988, see entire document.	1-9, 13-19, 40-42

☐ Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 1993	2 3 AUG 1993

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer KENNETH R. HORLICK
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